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## Opposite shell-coiling morphs of the tropical land snail *Amphidromus martensi* show no spatial-scale effects

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Much can be learned about evolution from the identification of those factors maintaining polymorphisms in natural populations. One polymorphism that is only partially understood occurs in land snail species where individuals may coil clockwise or anti-clockwise. Theory shows that polymorphism in coiling direction should not persist yet species in several unrelated groups of land snails occur in stably polymorphic populations. A solution to this paradox may advance our understanding of evolution in general. Here, we examine two possible explanations: firstly, negative frequency-dependent selection due to predation; secondly, random fixation of alternative coiling morphs in tree-sized demes, giving the impression of wider polymorphism. We test these hypotheses by investigating morph-clustering of empty shells at two spatial scales in *Amphidromus martensi* populations in northern Borneo: the spatial structure of snail populations is relatively easy to estimate and this information may support one or other of the hypotheses under test. For the smaller scale we make novel use of a statistic previously used in botanical studies (the K-function statistic), which allows clustering of more than one morph to be simultaneously investigated at a range of scales and which we have corrected for anisotropy. We believe this method could be of more general use to ecologists. The results show that consistent clustering or separation of morphs cannot be clearly detected at any spatial scale and that predation is not frequency-dependent. Alternative explanations that do not require strong spatial structuring of the population may be needed, for instance ones involving a mechanism of selection actively maintaining the polymorphism.

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Persistent polymorphisms in natural populations have long attracted interest from ecologists and evolutionary biologists because they may provide insights into important processes such as the evolution of disparate behavioural strategies, sympatric speciation, or frequency-dependent natural selection (for respective examples see Sinervo and Lively 1996, Seehausen and van Alphen 1998, Gigord et al. 2001). Land snails have

always figured prominently in this field of research since they often display conspicuous shell polymorphisms and are easily sampled, marked/recaptured and manipulated. Most land snail polymorphisms concern shell colour, shape and sculpture, which are demonstrably influenced by external factors such as predation (Cain and Sheppard 1954) and microscale vagaries of the abiotic environment (Johannesson et al. 1995). However,

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one particular class of land snail polymorphism remains only partially explained. Certain species of pulmonate snails display intraspecific polymorphism in coiling direction (chirality), with individuals expressing either clockwise (dextral) or anti-clockwise (sinistral) coiling of body and shell (reviewed in Schilthuizen and Davison 2005). In some cases this polymorphism is restricted to local populations (e.g. in *Lymnaea*) or can be explained by the existence of a cline between dextral and sinistral source populations (e.g. *Partula suturalis*, Johnson 1982). In other species, however, there exists a true polymorphism in which sinistral and dextral forms occur at appreciable frequencies more generally throughout a large population.

Coiling direction in pulmonates (which are hermaphroditic) is known to be determined by a single, highly conserved gene with alleles for sinistrality and dextrality, either of which may be dominant. The gene is expressed not in the bearer itself but in its eggs, resulting in delayed maternal inheritance (Sturtevant 1923, Boycott and Diver 1923, Degner 1952, Murray and Clarke 1976, Freeman and Lundelius 1982, Hierck et al. 2005). As coiling direction influences the entire anatomy of these animals, sinistral individuals carry their genital pore on the left-hand side of the head, whereas dextrals have it on the right-hand side. In species with globular shells, this difference is known to prevent mating between individuals with opposite coil (Gittenberger 1988). In species with taller shells, however, the bodies can be aligned, and two mirror-image individuals can still achieve successful copulation, albeit with difficulty in most species so far observed (Asami et al. 1998, but see Schilthuizen and Davison 2005). This behavioural reproductive isolation may in some cases act as a possible speciation mechanism (Gittenberger 1988, Ueshima and Asami 2003, Davison et al. 2005, but see Johnson et al. 1990).

Theoretical considerations and computer simulations show that the obstacles against interchiral mating will normally pose a disadvantage to any sinistral individual appearing in an otherwise dextral population, and vice versa (Orr 1991, van Batenburg and Gittenberger 1996, Stone and Björklund 2002) and even when interchiral mating is not impeded, monomorphism is the expected outcome in finite populations due to simple population genetic processes (Schilthuizen et al. unpubl.). Stable polymorphism is not expected.

It is surprising, therefore, that in at least thirteen unrelated genera of pulmonate land snails, intrapopulational coil polymorphism appears to be widespread (listed in Asami et al. 1998). In this study we focus on one of these genera, the southeast-Asian *Amphidromus*, specifically the subgenus *Amphidromus s.str.*, the representatives of which are common tree-dwelling species of primary and secondary tropical forests and in which coil polymorphism is common (Laidlaw and Solem 1961,

Sutcharit and Panha 2006). Several explanations may be offered for this polymorphism, some of which (linkage of the coiling gene to another gene that is under divergent selection; presence of sibling species; high rate of recurrent mutation between the two coiling alleles, etc.) are too specific to be applicable to the wide variety of polymorphic species in the genus. Some hypotheses allow us to make inferences about the spatial structure we may observe when sampling. The conspicuousness and low mobility of land snails makes it easy to estimate the spatial structure of their populations and so this gives us a ready means to investigate those hypotheses with a spatial element. We wish to highlight two such hypotheses here that appear plausible and testable.

Firstly, negative frequency-dependent selection imposed by predators may be invoked. If predators preferentially take the commoner morph, this provides a selective advantage for the rare morph, until the rare morph becomes common enough for the preference to be reversed. It is entirely plausible that predators such as birds can visually differentiate between dextral and sinistral morphs and may settle into a pattern of prey-handling that is suited for one morph only (for example, see the lateralisation of tool manufacture shown by New Caledonian crows, Hunt et al. 2001). Similarly, manually hunting small mammals such as primates may specialise in a prey handling strategy that benefits one coiling morph. Under this hypothesis, aggregation of coiling morphs may be expected at a spatial scale the size of predators' home ranges. Alternatively, the mechanism by which a predator captures its prey may itself be chiral with different rates of success when attacking sinistral and dextral snails. Some crabs such as the pebble crab *Eriphia smithii* show handedness in the relative size of their two claws, leading to differential success when preying on left or right-coiling snails but, interestingly, only when the prey species is high-spined (Shigemiyama 2003). Shell chirality of pond snails is also known to affect the success of those aquatic scavengers possessing asymmetric mandibles (Inoda et al. 2003).

Secondly, under a strongly subdivided population structure, apparent intrapopulational polymorphism may actually be composed of a mosaic of demes, each fixed for one coiling morph on a very small spatial scale. In these tree-dwelling, relatively sessile molluscs, migration between neighbouring areas may be limited, leading to the random fixation of alternative coiling morphs in each of many small demes. Recent evidence from the related species *Amphidromus inversus* in the Malaysian Peninsula shows that individuals move frequently between trees in a clump and can cross forest gaps between separated groups of trees (Schilthuizen et al. 2005). This appears to make it unlikely that *Amphidromus* populations are subdivided at a small spatial scale but does not rule out structuring at a slightly larger scale. The advantage of the analytical technique used in this study

is that it allows us, in effect, to scan through a range of spatial scales so that if there is a scale at which the chiral morphs are expressing a spatial structure, we are likely to find it.

In a first attempt to test these two hypotheses, we studied the distribution of coiling morphs in one *Amphidromus* species at small and large spatial scales. Clustering of coiling morphs at a small spatial scale, but not at the larger spatial scale of the home ranges of vertebrate predators, would support the hypothesis of polymorphism due to population structure, whereas the reverse would be support for the hypothesis of polymorphism due to negative frequency-dependent selection.

## Methods

### Study species

For this study, we selected the species *Amphidromus martensi* (Fig. 1). *Amphidromus martensi* is endemic to the island of Borneo, known from the Malaysian states of Sabah and Sarawak, and the Indonesian province of East Kalimantan (Laidlaw and Solem 1961). It is a common inhabitant of primary dipterocarp rainforest, where it is a forest canopy herbivore (Schilthuizen et al. unpubl.). Although the empty shells are common on the forest floor, living individuals are almost impossible to find without tree-climbing techniques. For this reason our study was conducted using empty shells only. Although direct observations of predation are not available, its traces (repaired tooth-shaped punctures on living individuals and fresh, empty shells with top whorls or one side of the shell having been forcibly broken off) suggest that small tree-dwelling mammals such as squirrels or rats may be important predators.



Fig. 1. A sinistral (left) and dextral (right) shell of *A. martensi* from Sabah, Borneo. Shell height is ca 40 mm. Apparent differences in shell shape are coincidental. The shells are greenish yellow with purplish bands.

### Large spatial scale

To study dextral-sinistral distribution on a large spatial scale, we sampled shells in the following seven lowland (<300 m a.s.l.) forest localities in the Malaysian state of Sabah, each measuring between 0.04 and 1.00 km<sup>2</sup>: Danum Valley Conservation Area (Danum; 4°58'N, 117°48'E), Gomantong Caves (Gomantong; 5°31'N, 118°04'E), Keruak Forest Reserve (Keruak; 5°31'N, 118°17'E), Pangi Forest Reserve (Pangi; 5°32'N, 118°18'E), Village of Sukau (Sukau; 5°30'N, 118°17'E), Tabin Wildlife Reserve (Tabin; 5°19'N, 118°44'E), and Tomanggong Hill (Tomanggong; 5°31'N, 118°18'E), (Fig. 2). In each forest we collected shells as they were encountered and the proportion of both coiling morphs was recorded. These proportions were tested against each other using pairwise proportion tests with sequential Bonferroni correction for multiple comparisons. They were subsequently tested individually against a ratio of 50:50 using exact binomial tests.

### Frequency of predation

After examining a large number of shells, we defined predation damage by the presence of small, neat holes (often repaired) in the shell, removal of the spire of the shell leaving the larger whorls intact, or damage to the mouth of the shell likely to be caused by biting. We excluded shell fragments or any shells where damage had clearly resulted from other causes (e.g. falls). We used  $\chi^2$  tests to compare numbers of predated and non-predated shells by chirality for the four locations (Sukau, Keruak, Pangi and Tomanggong) for which we have these data.

### Small spatial scale

To study spatial patterns of coil morph distribution on the small spatial scale, we set up five 20 × 20 m quadrats in suitable habitat: two at Keruak (Keruak 1 and 2), two at Tomanggong (Tomanggong 1 and 2) and one at Sukau (Sukau), (Fig. 2). Each of the quadrats was searched intensively by two or three persons, and the positions of all *A. martensi* shells and shell fragments were recorded with 0.1 m precision.

We were interested in patterns of aggregation or segregation between morphs at a range of spatial scales up to a maximum separation defined by the size of quadrat used. The more familiar methods of identifying deviation from complete spatial randomness, such as the index of dispersion and nearest neighbour method (applied to land snails in, for example, Schilthuizen and Lombaerts 1994, Ledergerber et al. 1997, Craze and Lace 2000) are not sufficiently flexible for this task,

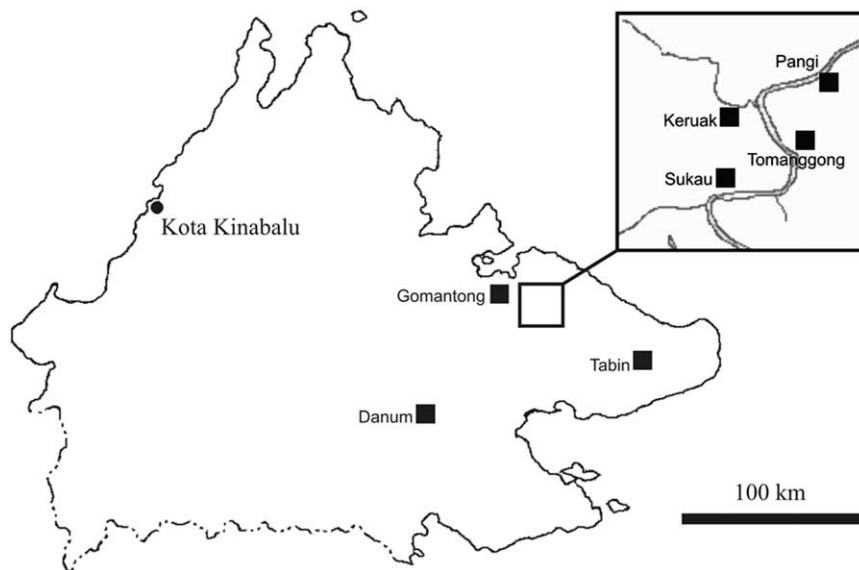


Fig. 2. Lowland forest locations in Sabah, Borneo, where sampling took place. The five  $20 \times 20$  m quadrats used for small-scale sampling were located at Keruak, Tomanggong and Sukau.

nor do they allow for the analysis of organisms classified into multiple states. Fortunately, the analysis of spatial point patterns is well advanced in the field of plant ecology and we have adopted a technique commonly used by botanists but not, to our knowledge, previously applied to land snails. The approach is to calculate the second-order or  $K$ -function statistic, as described by Ripley (1977) and extended to multiple state patterns by Upton and Fingleton (1985) [further details of all calculations are given in Diggle 1983]. For a given separation distance,  $r$ , and for each focal organism of class 1, the expected number of organisms of class 2 within a circle of radius  $r$  is calculated under the null hypothesis that the spatial pattern results from a Poisson process. The analysis is then repeated for focal organisms of class 2. The two resulting function estimates,  $K_{1,2}(r)$  and  $K_{2,1}(r)$ , are incorporated into a single estimator which has the expected value of  $r$  under the null hypothesis. For convenience, the value of this estimator is subtracted from  $r$  giving a function ( $L(r)$ ) with an expectation of zero. Large positive values of  $L(r)$  indicate that the two classes of organism are segregated at scale  $r$  while large negative values indicate aggregation. Underestimation of  $K(r)$  due to edge effects was corrected using a weighting procedure due to Ripley (1977).

## Programming

The analysis was carried out using programs specially written in R (ver. 2.1.1, R development Core Team). These incorporated a function which calculates an estimate of  $K(r)$  given a set of classified points (Baddeley et al. 2005). These estimates of  $K(r)$  were used to derive

$L(r)$  with the significance of any deviation from complete spatial randomness tested using a Monte-Carlo procedure, as recommended by Upton and Fingleton (1985). This involved calculating estimates of  $L(r)$  for 100 randomisations of  $n_1$  points of class 1 and  $n_2$  points of class 2. The 95% confidence limits shown in the results were then calculated from the resulting distribution of estimates.

In some cases, significant aggregation or segregation was indicated but this may have been due to inhomogeneity in the spatial distribution of shells rather than any tendency of sinistrals or dextrals to interact (i.e. due to anisotropy). To test this we ran a second program in which the positions of the shells were held constant but their classification as sinistral or dextral was randomly assigned, within the constraint that the total number of each was the same as that actually observed. Again, 100 randomisations were carried out and 95% confidence intervals calculated. In effect this allows us to compare the actual distribution of morphs with random allocations having the same degree of anisotropy.

In a few cases, fragments of shells could not be unambiguously classified as either dextral or sinistral. Rather than remove these fragments from the analysis, we carried out a number of runs of both programs with the set of unclassified fragments having all possible assignments of morph so as to determine the maximum influence they might have on the resulting estimates of  $L(r)$ .

Frequencies of sinistral and dextral shells in each of the five quadrats were examined using binomial tests first against a ratio of 50:50 and then against the observed overall ratio at that site determined from the large-scale data.

# Results

## Large spatial scale

Comparison of proportions between sites showed a general pattern of similarity but with Tabin appearing to be different from Gomantong and Sukau (binomial tests:  $p=0.014$  and  $p=0.012$  respectively) and Danum being just significantly different from Sukau (binomial test:  $p=0.049$ ). However, none of these differences remained significant after Bonferroni correction. Subsequent individual binomial tests against a theoretical ratio of 50:50 sinistral to dextral showed that the Tabin sample had significantly more sinistral shells than expected (Table 1). Both Sukau and Gomantong have high proportions of dextrals and, while these are not significantly different from 0.5, they may account for the weak evidence of a difference in proportion between these sites and Tabin. The mean dextral proportion across all sites is 0.480 (95% CI 0.443–0.517,  $n=7$ ).

## Frequency of predation

Chirality has no effect on the probability that a shell will show predation damage (Table 2). If the observed trends continued, the result for Sukau might possibly become significant with a sample size that is reasonable for the area but other required sample sizes are rather higher making it unlikely that any of these are biologically important deviations from equal frequency of predation.

## Small spatial scale

For the five quadrats sampled, locations of sinistral, dextral and unidentified shells are shown in Fig. 3. None suggest any obvious pattern with respect to morphs although one, Keruak 1, shows some non-randomness in the overall distribution of *A. martensi*. Accompanying these distribution maps, in Fig. 4 and 5, are the results of K-function analysis, with the derived L-function

plotted against separation distance. Figure 4 shows the L-function against the results of randomisation while Fig. 5 shows the L-function against the results of randomisation of morph with point locations held constant.

In no case is there any clear evidence that sinistral and dextral shells of *A. martensi* show a tendency to segregate or aggregate at any spatial scale defined by the quadrats used in this study. In Keruak 1 there is an apparent aggregation of sinistrals and dextrals but this is clearly due to the inhomogeneity in the distribution of shells regardless of morph and the effect is removed when the location of shells is held constant (Fig. 5).

The L-function derived from shells in Keruak 2 falls entirely within the 95% confidence limits for both analyses and therefore shows no tendency for segregation or aggregation.

The very unequal numbers of sinistral and dextral morphs found at sites Sukau and Tomanggong 1 make the results less reliable and increase the probability of a random pattern being interpreted as aggregation. This is, in fact, what appears to have happened in these cases. The apparent significant clumping of morphs at short separations is probably an effect of the tight clumping of one sinistral and two dextral shells in Tomanggong 1 and one sinistral and one dextral shell in Sukau. The effect is removed when shell locations are held constant (Fig. 5).

There appears to be a just significant segregation of morphs in Tomanggong 2 at a separation of just under 1 m. However, this is not significant compared to randomly located shells and is probably due to four sinistral shells forming two pairs.

When examining the frequencies of sinistrals and dextrals in individual quadrats, only Tomanggong 1 was found to have a ratio of sinistrals to dextrals significantly different from 50:50 (in this case, having significantly more dextrals) (Table 3). The ratio for this quadrat also differed significantly from the empirical ratio for the Tomanggong site as a whole. Some of the quadrats yielded relatively small numbers of shells and so we have used power analysis for proportion tests to

Table 1. Results of testing the observed proportion of sinistrals and dextrals at each site against a theoretical ratio of 50% sinistral, 50% dextral and against the observed proportion of dextrals for all sites sampled. P(D) is the observed proportion of dextrals; 95% CI is the 95% exact binomial confidence interval. \* = significant at 0.05.

Site	Number of:		p(D)	95%CI	p for $H_0: p(D)=0.500$	p for $H_0: p(D)=0.480$
	Dextrals	Sinistrals				
Danum	29	43	0.403	0.289–0.525	0.125 (NS)	0.197 (NS)
Tabin	17	34	0.333	0.208–0.479	0.024*	0.049*
Gomantong	56	44	0.560	0.457–0.659	0.271 (NS)	0.111 (NS)
Tomanggong	42	48	0.467	0.361–0.575	0.595 (NS)	0.833 (NS)
Pangi	50	56	0.472	0.374–0.571	0.627 (NS)	0.923 (NS)
Sukau	37	26	0.587	0.456–0.710	0.207 (NS)	0.101 (NS)
Keruak	112	121	0.481	0.415–0.547	0.600 (NS)	1.000 (NS)
All sites	343	372	0.480	0.443–0.517	0.295 (NS)	NA

Table 2. Numbers of predated and non-predated shells in areas sampled. See the text for definition of what constitutes predation damage. Also shown are the results of Chi-squared contingency table tests and the required sample sizes required to make the observed differences just significant with power = 0.80 at  $\alpha = 0.05$ .

Site	Chirality	Predated	Not predated	$\chi^2$	p	Sample size for sig. diff.
Sukau	D	6	15	1.05	0.305	164
	S	10	13			
Keruak	D	30	53	1.42	0.233	332
	S	22	25			
Pangi	D	24	19	0.28	0.596	1162
	S	20	20			
Tomanggong	D	9	9	0.17	0.678	928
	S	13	10			

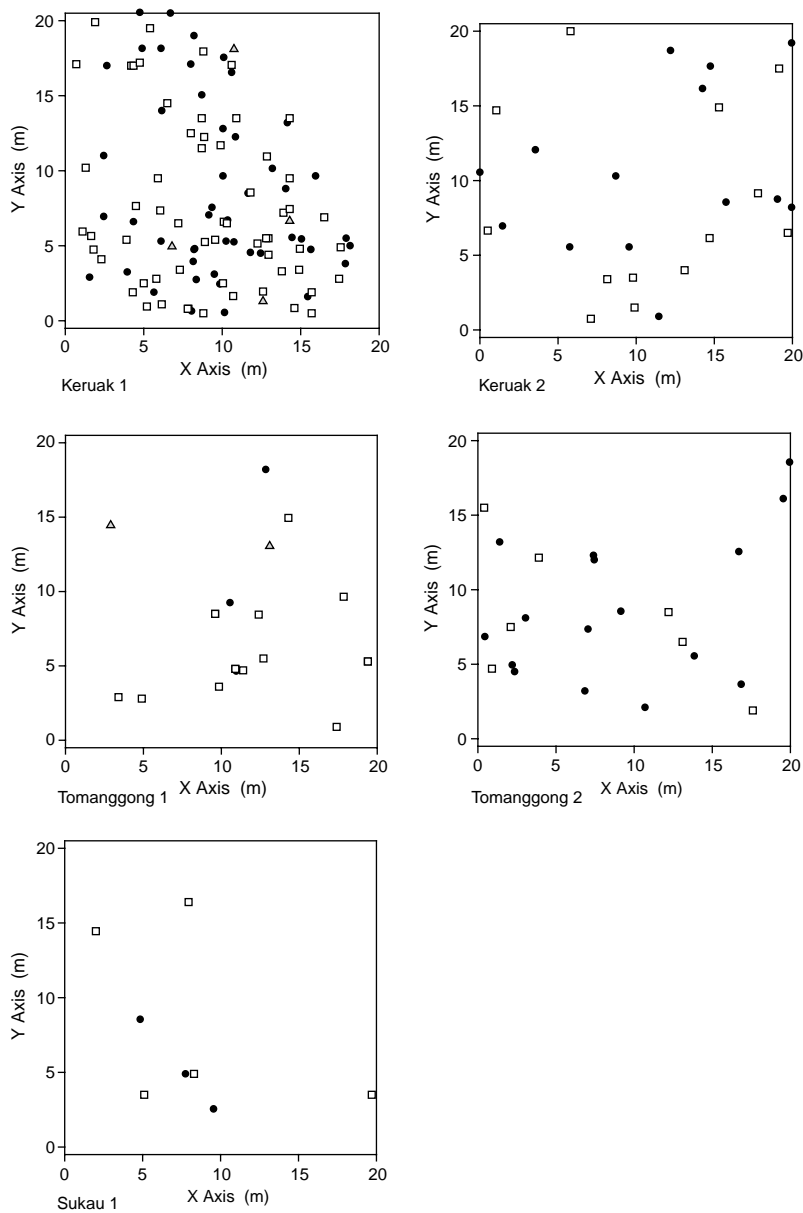
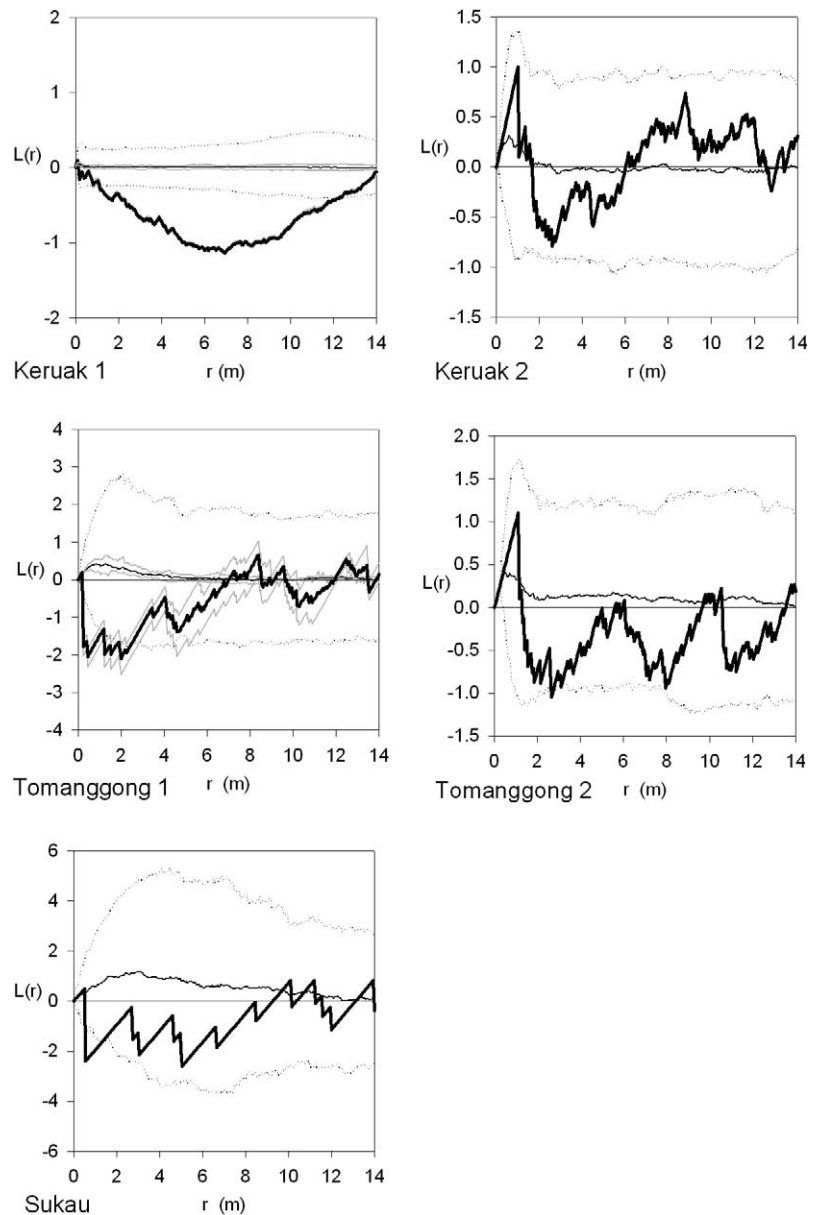


Fig. 3. Location of sinistral (black circles) and dextral (open squares) shells and shell fragments in the searched quadrats. Locations of shell fragments that could not be identified as sinistral or dextral are also shown (grey triangles).

Fig. 4. L-function against separation distance thick, black line. Where unidentified shell fragments were present, the extent of their effect is shown by the pair of thin, grey lines accompanying the main line. Also shown is the mean L-function for the 100 randomisations thin, black line and the associated 95% confidence limits dotted lines. Again, the maximum effect of unidentified fragments on the mean line is shown by grey lines. In all cases the influence of these unidentified fragments is minimal.



calculate the sample size needed to make the observed non-significant ratios just significant (Table 4). With power = 0.80, only Tomanggong 2 could be made significant at  $\alpha = 0.05$  with reasonable sample sizes (the result for Sukau is unlikely to be reliable due to the particularly small sample size). This result is especially interesting given that in Tomanggong 2, if the non-significance was indeed due to the small sample size, sinistrals may be more numerous. This would then contrast with the situation in the nearby Tomanggong 1. The proportion of dextrals is, indeed, significantly different between the two quadrats based on the result of a binomial test ( $p < 0.001$ ).

## Discussion

The essential premise of this study was that any observable patterns in the spatial distribution of morphs might reveal something of the underlying factors responsible for the maintenance of coiling polymorphism in this species. Using data from previous collections, the overall ratio of sinistral to dextral morphs was expected to be ca 50:50. Departures from this ratio might occur at any of a range of spatial scales such that we might be able to infer evidence for or against several competing hypotheses put forward to explain the coiling polymorphism. However, the most general



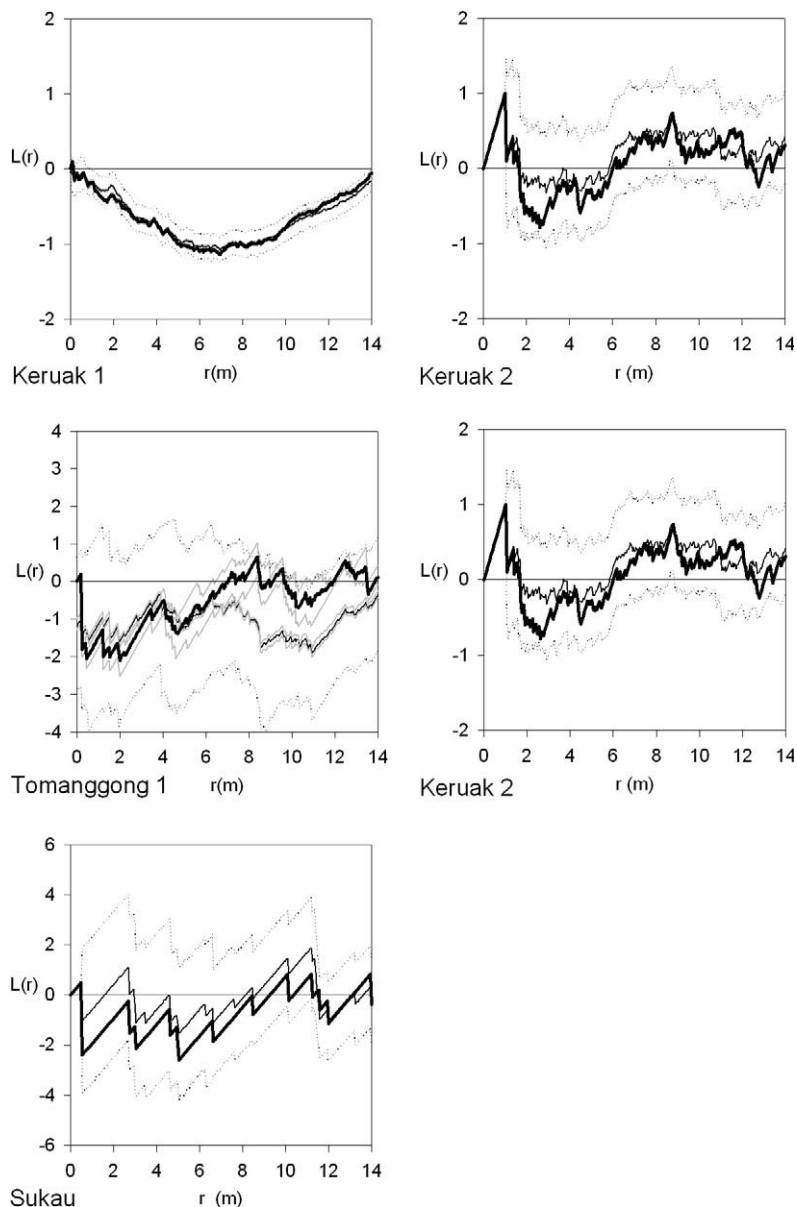


Fig. 5. As Fig. 4 but here the randomisations are carried out by holding the locations of the shells constant and randomly defining them as sinistral or dextral. This corrects for anisotropy in the overall distribution of shells.

result from this study is that there is little, if any, consistent departure from a ratio of 50:50 at any of the spatial scales examined. We therefore have no clear evidence that the population of *Amphidromus martensi* exists as a mosaic of sinistral and dextral sub-populations either at the scale of individual trees or at a scale relevant to a possible predator species.

Although found to be non-significant after Bonferroni correction, it is possible that the proportion of dextrals found at Tabin is lower than that at some other areas and lower than the expected 0.5. A more extensive survey of sites across Sabah with larger samples collected

at each area would be needed to confirm or refute this non-significant trend.

There was little suggestion that the population was structured at the scale of individual trees. However, in at least one case (Tomanggong 1) there was evidence for a departure from a ratio of 50:50 at the scale of  $20 \times 20$  m quadrats. In the next nearest quadrat (Tomanggong 2, at a distance of  $<100$  m) the ratio of morphs appears to be reversed, although the small sample size makes this second finding uncertain. This observation is almost the only indication of spatial structuring at a small scale and therefore deserves proper consideration but it must be

Table 3. Results of binomial tests for each of the five quadrats sampled. Proportions are given in terms of the frequency of dextrals with  $p(D)_q$  being the observed proportion of dextrals in the quadrat and  $p(D)_s$  being the observed frequency of dextrals in the site as a whole. The values of  $p(D)_q$  are tested first against a theoretical proportion of 0.5 and then against  $p(D)_s$ . The two lines of data for Keruak 1 and Tomanggong 1 show the effects on the results of calling unclassified fragments dextral or sinistral. \* = significant at 0.05; \*\* = significant at 0.01.

Site	Number of:		$p(D)_q$	95%CI	p for $H_0: p(D)_q = 0.5$	$p(D)_s$	p for $H_0: p(D)_q = p(D)_s$
	Dextrals	Sinistrals					
Keruak 1	66	50	0.569	0.474–0.661	0.163 (NS)	0.481	0.063 (NS)
Keruak 1	62	54	0.534	0.440–0.628	0.516 (NS)	0.481	0.265 (NS)
Keruak 2	15	16	0.484	0.302–0.669	1.000 (NS)	0.481	1.000 (NS)
Tomanggong 1	15	5	0.750	0.509–0.913	0.041*	0.467	0.013*
Tomanggong 1	17	3	0.850	0.621–0.968	0.003**	0.467	0.001**
Tomanggong 2	7	16	0.304	0.132–0.529	0.093 (NS)	0.467	0.144 (NS)
Sukau	5	3	0.625	0.245–0.915	0.727 (NS)	0.587	1.000 (NS)

Table 4. The required total sample sizes needed to make the observed results statistically significant at  $\alpha = 0.05$  with power = 0.8 for the five quadrats sampled. Only the result for Tomanggong could be made significant with a reasonable sample size if current trends continued. As with Table 3, the two lines of data for Keruak 1 and Tomanggong 1 show the effects on the results of calling unclassified fragments dextral or sinistral.

Site	Sample size for sig. diff	
	against $p(D)_q = 0.5$	against $p(D)_q = p(D)_s$
Keruak 1	424	265
Keruak 1	1704	713
Keruak 2	7744	218000
Tomanggong 1	N/A	N/A
Tomanggong 1	N/A	N/A
Tomanggong 2	51	70
Sukau	134	1331

seen in the context of all the evidence relating to *A. martensi* and *A. inversus*. The small sample size coupled with the fact that a similar pattern is not observed elsewhere and the evidence of movement between trees in *A. inversus* (Schilthuizen et al. 2005) suggests this observation is unlikely to be of biological significance in explaining morph pattern. Also, variation at this spatial scale may be indicative of negative frequency dependent selection due to predation. However, we found no further supporting evidence for this from our examination of numbers of predated versus non-predated shells.

While there are gaps in the available evidence and some indications that may be followed up in subsequent studies, the pattern that is emerging for both *Amphidromus martensi* and *A. inversus* is one of mixed populations with no obvious, consistent spatial or ecological separation by morph. Whatever factors exist to maintain the polymorphism, it seems likely they must operate without the need for strong spatial structuring of the population. We believe sexual selection for inter-chiral mating may be the most likely mechanism and we describe this in full elsewhere (Schilthuizen et al. unpubl., see also Schilthuizen and Davison 2005,

Davison et al. 2005). The confirmation of selection actively maintaining such a polymorphism may be significant.

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